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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Dated: April 2, 2004

WEI HUANG, MERL F. HOEKSTRA,
J. RICHARD SPORTSMAN,
and EWALD A. TERPETSCHNIG

Examiner Changhwa J. Cheu

Serial No. : 09/844,655

Group Art Unit 1641

Filed : April 27, 2001

For : MOLECULAR MODIFICATION ASSAYS

U.S. Patent and Trademark Office
Attention: Changhwa J. Cheu
Group Art Unit 1641
Washington, D.C. 20231

Sir:

DECLARATION OF J. RICHARD SPORTSMAN UNDER 37 C.F.R. § 1.132

I, J. RICHARD SPORTSMAN, declare:

1. I am one of the inventors named on U.S. Patent Application Serial No. 09/844,655.

2. I am currently an employee of Molecular Devices Corporation ("MDC"), which is the assignee of the '655 application. I currently hold the position of Vice President, Assay and Reagent Research and Development, at MDC. If called as a witness, I could competently testify to the following facts, all of which are within my own personal knowledge.

General Background

3. I am trained as an analytical biochemist. I received a Ph.D. in Analytical Chemistry from the University of Arizona in 1982, and I completed post-doctoral studies in Clinical Immunology at Scripps Clinic and Research Foundation in 1984.

4. I have worked at several biotechnology or pharmaceutical companies, including Eli Lilly and Company, SyStemix, Inc., and Terrapin Technologies (now Telik). Each of these companies is or was involved in drug discovery or development of human therapeutics. In my work at these companies, I have acquired special expertise in methods of drug discovery using binding tests ("assays") based on fluorescence polarization.

5. I have been employed by MDC or LJL Biosystems ("LJL") since August 1998. In August 1998, I joined LJL, which merged with MDC two years later in August 2000. At LJL and MDC, I have been responsible for research on various chemical tests and the development of kits that use such tests.

6. Further details of my employment history and qualifications are described in my resume, which is attached as an Appendix of this Declaration.

Comparison of Iron and Gallium in Binding Assays

7. I designed and conducted a series of binding assays to compare the metals iron ("iron reagent") and gallium ("gallium reagent"). Each binding assay included either a fluorescent substrate (a nonphosphorylated peptide) or a fluorescent product (a phosphorylated form of the peptide). After incubation of the fluorescent substrate or product with the iron reagent or the gallium reagent, I measured the effect of each metal on luminescence intensity and polarization from the substrate or product. Accordingly, these binding assays with gallium correspond to an embodiment of the invention for detecting kinase enzyme activity in which there is no enzyme activity (substrate (S) only) or substantial enzyme activity (product (P) only). Alternatively, by reversing the substrate and product designations of these peptides, the results correspond to an

embodiment of the invention for detecting phosphatase enzyme activity in which there is substantial enzyme activity or no enzyme activity, with similar conclusions.

I. Reagents

8. I used the following reagents for the binding assays. Binding buffer was prepared as an aqueous solution of 50 mM acetic acid and 500 mM NaCl, titrated to pH 5.0 with NaOH. Concentrated stock solutions of the metals were prepared as 0.67M $FeCl_3$ or 0.67M $GaCl_3$ in 0.1 M HCl. The iron reagent and gallium reagent were prepared for addition to binding assays by 400-fold dilution of each respective, concentrated metal stock solution with the binding buffer. Reaction buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1% BSA, 0.05% NaN_3, pH 7.2) was used to prepare 100 nM solutions of fluorescent peptides for the binding assays.

9. The fluorescent peptides used for the binding assays have amino acid sequences corresponding to a fragment of glycogen synthase. These peptides were synthesized chemically and have the following sequences: (1) 5FAM-Lys-Lys-Leu-Asn-Arg-Thr-Leu-Ser-Val-Ala (substrate, "S"), and (2) 5FAM-Lys-Lys-Leu-Asn-Arg-pThr-Leu-Ser-Val-Ala (product, "P"). In these sequences, "5FAM" represents a 5-carboxy fluorescein moiety (a luminophore) covalently linked to the amino terminus of each peptide, and pThr is phosphothreonine. The substrate (S) and product (P) peptides differ only by the absence (S) and presence (P) of a phosphate group on threonine. The substrate peptide has been used successfully in kits sold by Molecular Devices Corporation to test the activity of MAPKAP K2, PRAK, and CaM KII enzymes using the gallium reagent in fluorescence polarization assays.

II. Performance of Binding Assays

10. Binding assays were performed using the reagents described above in Section I. Binding reactions were assembled by combining 60 µL of the iron reagent or the gallium reagent with 20 µL of 100 nM S or P peptide in wells of a microplate. Each binding reaction was incubated for thirty minutes at room temperature. Then, fluorescence polarization was measured on an Analyst ® AD instrument, which is available commercially from Molecular Devices Corporation. In particular, each binding reaction was illuminated with polarized light of 490 nm, and component fluorescence intensities were measured from directions parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the direction of polarization for the incident polarized light.

11. Fluorescence polarization (FP) was calculated according to the following formula, where G is an instrument factor:

$$FP \text{ (in mP)} = 1000 \times (I_{||} - G \times I_{\perp}) / (I_{||} + G \times I_{\perp})$$

Total fluorescence intensity (I_{TOT}) was calculated according to the formula:

$$I_{TOT} = I_{||} + 2 \times I_{\perp}$$

Each combination of reaction components was assayed in triplicate, and standard deviations were calculated based on these triplicate assays.

III. Results of Binding Assays

12. Figure 1 shows results of total fluorescence intensity measurements in relative fluorescence units (RFU), as a function of metal (iron reagent or gallium reagent) and peptide phosphorylation state (P or S) in the binding assays.

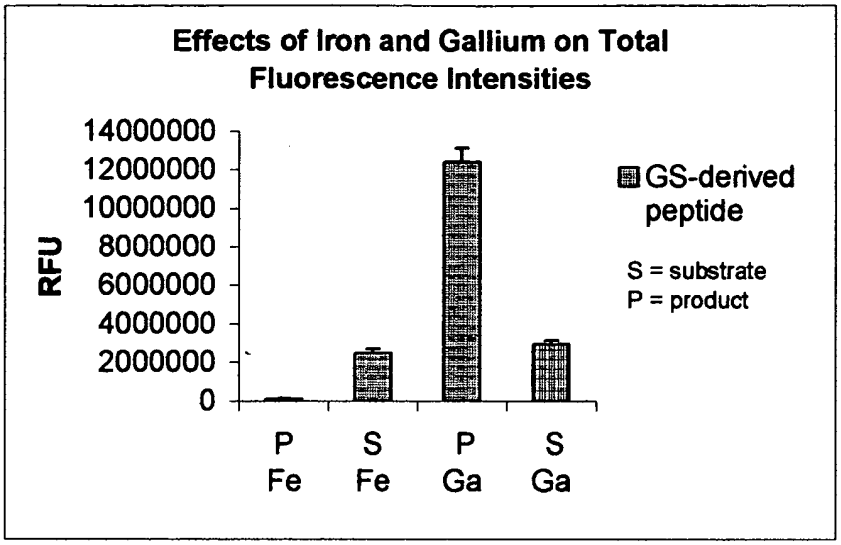

Figure 1

The fluorescence intensity was generally comparable in assays of the substrate (S) performed with either the iron reagent or the gallium reagent. This result is consistent with little or no binding of each reagent to this substrate, and thus little or no effect on the brightness of the luminophore associated with this substrate. However, the fluorescence intensity or brightness differed dramatically according to added metal (iron or gallium) in assays of the product (P). In particular, the brightness was about one-hundred-fold higher in assays of the product performed with the gallium reagent relative to the iron reagent. This difference in brightness was produced by an approximately four-fold increase in the brightness of the product relative to substrate for the gallium reagent, and by a more than twenty-fold decrease in this brightness relative to substrate (S) for the iron reagent. Therefore, the iron reagent quenched the brightness of the product substantially, while the gallium reagent not only did not quench but actually enhanced this brightness.

13. Figure 2 shows a plot of fluorescence polarization (mP) measured as a function of added metal and peptide phosphorylation state.

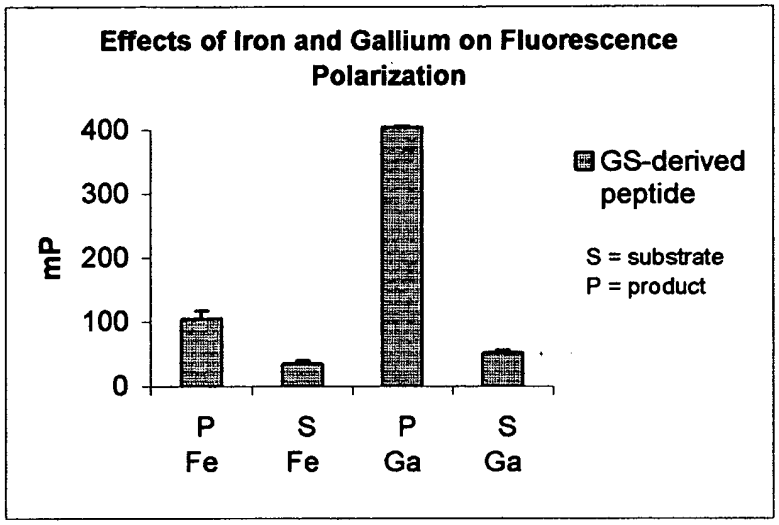

Figure 2

The difference between the degree of polarization produced by luminophore-associated product and substrate defines the "dynamic range" of these assays. In the present configuration, the iron reagent provides a dynamic range of about 70 mP, and the gallium reagent a dynamic range of about 350 mP.

Statement Under 18 U.S.C. §1001

14. I hereby declare that all statements made herein of my own knowledge are true and that statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United State Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: April 2, 2004

J. RICHARD SPORTSMAN

CURRICULUM VITAE

J. Richard Sportsman, Ph.D.

Vice President, Reagent and Assay
Research and Development
Molecular Devices Corporation
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Research Interests:

Molecular recognition for analysis and drug discovery; molecular immunology; instrumental analysis; fluorescence methods in quantitative biology; high throughput screening.

Education:

Post-doctoral Fellow in Clinical Immunology Scripps Clinic and Research Foundation, La Jolla, California 1982 - 1984

Ph.D. in Analytical Chemistry
Department of Chemistry
University of Arizona, Tucson, Arizona 1982.

A.B. in Chemistry
Occidental College, Los Angeles, California 1975

Positions:

Vice President, Assay and Reagent Research and Development	August 2002 to present
Director of Biochemistry	August 2000 to July 2002
Senior Director, Assay Systems LJL Biosystems	August 1998 to August 2000
Staff Scientist and Director of Molecular Recognition Telik, Inc. (formerly Terrapin Technologies, Inc.)	September 1993 to July 1998
Manager of Biological Chemistry SyStemix, Inc	August 1992 to September 1993
Department of Biotechnology Research Lilly Research Laboratories, Inc.	September 1984 to July, 1992
Analytical Research Laboratories, Inc.	November, 1976 to

Monrovia, CA	August, 1977
Truesdail Laboratories, Inc. Los Angeles, CA	December, 1974 to May, 1976

Publications:

1. Sportsman JR, Wilson GS (1980) "Chromatographic properties of silica-immobilized antibodies" Anal Chem 52:2013-2018.

2. Sportsman JR, Liddil JD, Wilson GS, (1983) "Kinetic and equilibrium studies of insulin immunoaffinity chromatography" Anal Chem 55:771-775

3. Johnson DA, Gautsch JW, Sportsman JR, Elder JH (1984) "Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose" Gene Anal Techn 1:3-7.

4. Sportsman JR, Elder JH (1983) "A microanalytical protein assay using laser densitometry" Anal Bioch 139:298-302

5. Sportsman JR, Park MM, Cheresh DA, Fukuda M, Elder JH, Fox RI (1985) "Characterization of a membrane surface glycoprotein associated with T-cell activation" J Immunol 135:138-142

6. Fox RI, Sportsman JR, Rhodes G, Luka J, Pearson G, Vaughan JH (1986) "Rheumatoid arthritis synovial membrane contains a 62 kD protein that shares an antigenic epitope with the Epstein-Barr virus-encoded EBNA-1 antigen" J Clin Invest 77:1539-1543.

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for a Range of Protein Serine/Threonine and Tyrosine Kinases" J Biomol Screen 8: 165-75

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7. Sportsman JR, Kauvar LM, Villar HO, Spevak WR "Nonpeptide insulin receptor agonists" US patent 5,851,988 (1998)

8. Sportsman JR, Cairns N, (1997) "Competitive fluorescence polarization assay for kinases" US Patent pending.

9. Sportsman JR, Huang W, Terpetschnig E, Hoekstra M "Luminescence Kinase Assays" (2000) US Patent pending

Invited Lectures:

1. "Quantitation of the immune response to recombinant products" Seminar on Analytical Biotechnology, Baltimore, MD, May 17-20, 1988.

2. "New approaches to analyses for therapeutic compounds" Chemistry Department seminar, Occidental College, Los Angeles, CA, April 2, 1990.

3. "Fluorescence immunoassays for quantifying low molecular weight compounds in plasma at picomolar levels" Symposium on new immunoassay

methods, American Chemical Society Meeting, Division of Analytical Chemistry, Boston, MA April 23, 1990 Abstract #28.

4. "New approaches in hybridoma and drug screening programs" Data management technologies in biological screening: pharmaceutical and agrochemical discovery SRI International, Menlo Park, CA, April 24, 1992.

5. "Fluorescence Polarization Assays for High Throughput Screening".2nd European Conference on High Throughput Screening Budapest, Hungary, May 17, 1995

Presentations:

1. Sportsman, JR, Heisserman JA, Winely CW, Frank BH (1987) "Methionyl-human growth hormone provokes elevated levels of antibodies to human growth hormone relative to natural sequence human growth hormone." Poster presentation at Endocrine Society meeting, June 10, 1987, Indianapolis, Indiana

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4. Taber LD, Poor ML, Sportsman JR (1988) "Automated competitive ELISAs for determining low femtomole levels of therapeutic drugs" Paper presented at Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, February 23, 1988, New Orleans, Louisiana Abstract #334

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7. Nielsen RG, Sittampalam GS, Rickard EC, Smith DR, Taber LD, Sportsman JR Separation of immune complexes by capillary zone electrophoresis International Bioanalytical Workshop, University of Kansas, May 21, 1989, Lawrence, Kansas Poster P21.

8. Fernando SA, Winely CL, Sportsman JR, Wilson GS (1989) "Fundamental studies of the 'low dose hook effect' in a competitive homogeneous immunoassay" Presented at the 1989 International Bioanalytical Workshop, May 23, 1989, Lawrence, KS Abstract P19.

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14. Sportsman, JR (1994) "Drug Discovery through Molecular Fingerprinting" Presented at 1994 International Forum on Advances in Screening Technologies and Data Management, Princeton, New Jersey, April 18, 1994.

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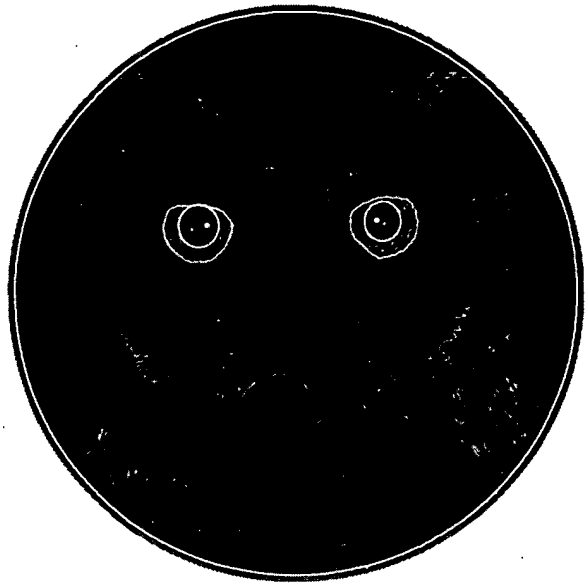

IQ®

Assay Platform

Technical Handbook

PIERCE
a Perbio Science Company

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CONTENTS

Introduction

IQ® Technology is designed for determination of protein kinase and phosphatase activity using a powerful homogeneous assay platform based on fluorescence intensity quenching. The assay incorporates an iron-containing compound that binds specifically to phosphate groups present on fluorescent dye-labeled phosphorylated peptides. When bound to the phosphate group, the iron-containing compound is brought into proximity to the fluorophore and acts as a dark quencher of the fluorescent dye. This specific binding is immediate. Results are quantitated by comparing the observed relative fluorescence units of test samples to blanks containing no enzyme. The results can be directly correlated to moles of phosphopeptide.

Fluorescence

Fluorescence is a type of photoluminescence, in which a photon of energy supplied by an external source is absorbed by a molecule, ion or atom, creating an excited electronic singlet state (S1). When the radiant energy of S1 is partially dissipated, a relaxed singlet excited state is yielded, producing a fluorescent emission. Fluorescent molecules have representative excitation and emission wavelengths. More in-depth background information on fluorescence is available in many textbooks.

Quenching

Many mechanisms can cause a decrease in fluorescence intensity, called quenching. In the IQ® Technology, quenching results from the binding of a proprietary iron-containing compound to phosphate groups in proximity to the fluorophore. The emitted energy is absorbed by the moiety, thus reducing the total fluorescence. IQ® Reagents bind specifically to the phosphorylated form of a peptide, so the proportion of phosphorylated to nonphosphorylated peptide in a system can be directly quantitated by the change in fluorescence intensity. Higher fluorescence intensity units correlate to little or no phosphorylated peptide, while low fluorescence intensity units correlate to high concentrations of phosphorylated peptide; therefore, the relationship between phosphorylation state and fluorescence intensity is inversely proportional. The specificity of the quenching to phosphorylated peptides will be shown empirically in a later section.

Fluorescence Intensity and IQ® Assays

Quantitation of kinase or phosphatase activity in the IQ® Assay is accomplished by measuring the change in fluorescent signal using a standard plate fluorometer. The change in fluorescent signal is associated with binding by the IQ® Reagent to phosphoryl groups in proximity to a fluorophore. Thus, the amount of signal quenching corresponds to the level of phosphorylated peptide in the reaction mixture. For kinase reactions, the fluorescence intensity will decrease in proportion to the percent phosphorylated product when compared to a no-enzyme control; for phosphatase reactions, the fluorescence intensity will increase in proportion to the percent de-phosphorylated product compared to a no-enzyme control (Figures 1A and 1B). Detection of the end product is measured directly.

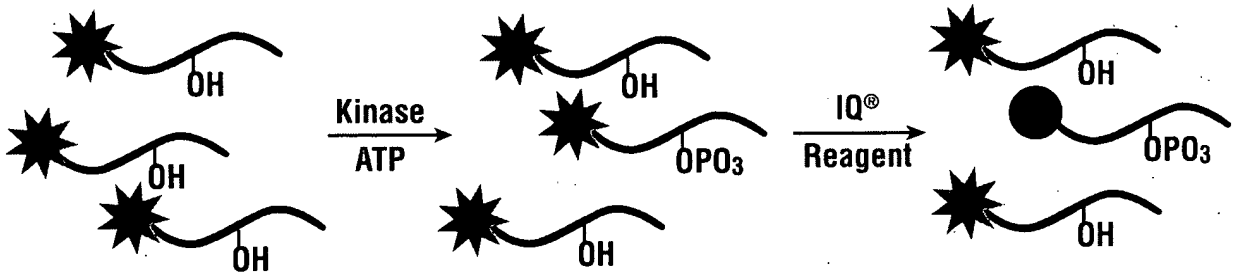

Figure 1A. Schematic depicting quenching of dye on peptides phosphorylated by a kinase using the IQ® Technology. For kinase reactions, the fluorescence intensity will decrease in proportion to the amount of phosphorylated product.

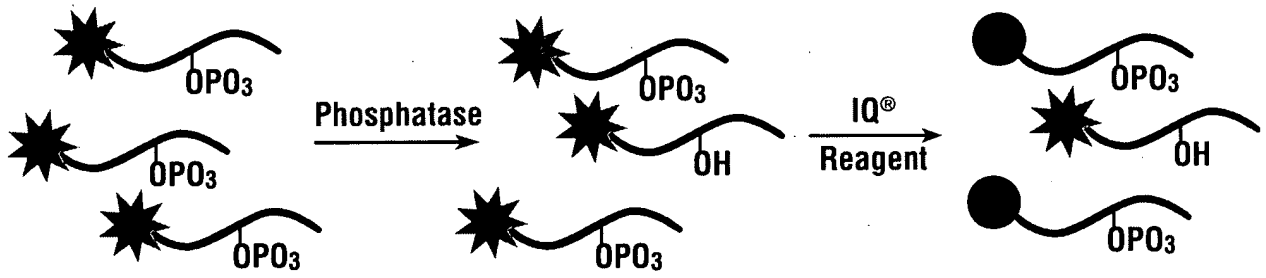

Figure 1B. Schematic depicting quenching of dye in a phosphatase assay only on phosphorylated peptides using the IQ® Technology. For phosphatase reactions, the fluorescence intensity will increase in proportion to the amount of de-phosphorylated product.

Fluorescent Dye Used with the IQ® Technology

The peptide substrates used in the IQ® Assay are labeled with a rhodamine derivative dye. Its spectrum is illustrated in Figure 2. The maximum absorbance is at a wavelength of 569 and maximum emission is at 592. Any plate fluorometer can be used to measure the fluorescence intensity of this dye. If using a filter-based instrument, the optimal filter set for this dye is a 560 excitation/590 emission with narrow bandwidths, such as 7 nm. Some other filter sets may be used, as long as the instrument parameters are set to eliminate the majority of excitation light from the emission measurement through the use of dichroic mirrors, emission filters and bandwidth selection. Examples of measurement comparisons using other filter sets are indicated in Figure 3.

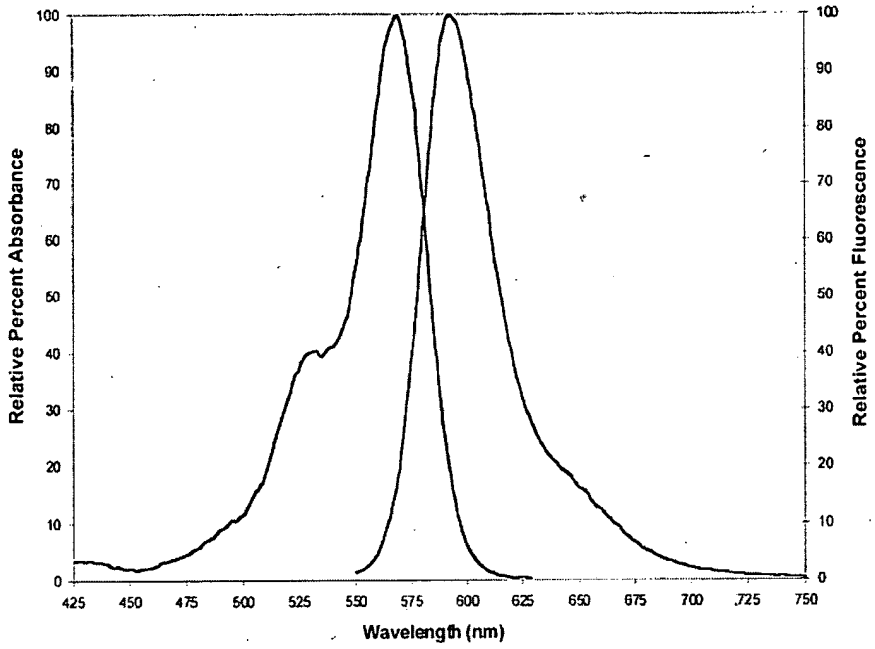

Figure 2. Excitation and emission profile of rhodamine derivative-labeled peptide used in IQ® Assays (569/592 Ex/Em Peak).

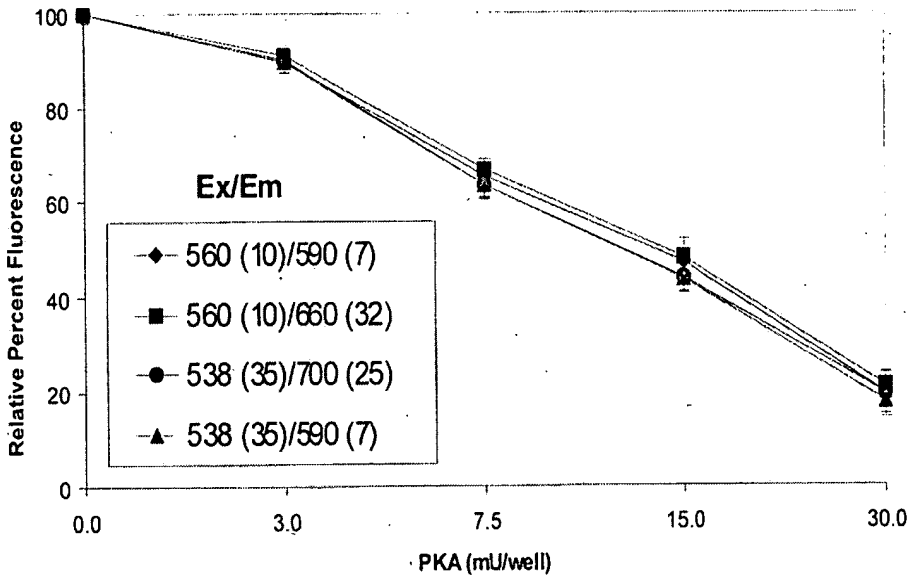

Figure 3. Comparison of different filter sets for use in IQ® Assays. PKA was titrated at five different concentrations and the reaction allowed to proceed for 1 hour, then IQ® Reagents were added. The plate was evaluated using the various filter sets, and data were normalized versus a no-enzyme control. At increasing concentrations of enzyme, the fluorescence decreases. This correlates to the increased amount of phosphopeptide generated. Normalized data were obtained for each filter set used and indicate equivalent results.

Plate Selection for use in IQ® Assays

Generally black plates are recommended for measuring fluorescence, due to low background fluorescence from the plate itself. However, white plates may also be used in fluorescence-based assays that rely on simple fluorescence intensity measurement, because the differences in noise can be normalized out of the final data. Therefore, any opaque plate may be used in the IQ® Assays. One advantage of using a white plate in this application is to permit observation of the colored fluorophore within the wells and to avoid manual pipetting errors. A comparison of relative percent fluorescence using 384-well white or black plates is illustrated in Figure 4. A general comparison of multiple plate types used in an IQ® Assay resulted in no difference in measured enzyme activity. Although absolute values in the raw data will vary from plate-to-plate, the percent product formed can be accurately measured using most plate types by converting the raw data to relative percent fluorescence (see Data Management section).

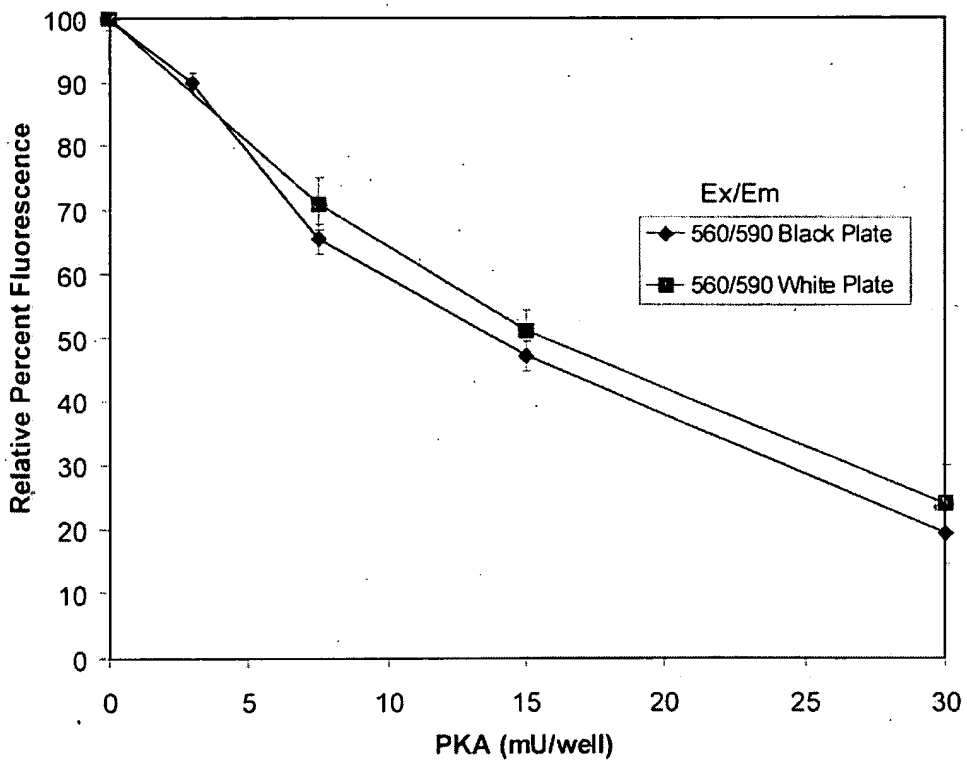

Figure 4. Comparison of PKA detection in 384-well white and black plates. PKA was titrated at six concentrations into either white or black plates, and the reaction was allowed to proceed for 1 hour. IQ® Reagents were added and the fluorescence was measured. Normalized data indicate no significant difference in results.

Plate Density in the IQ® Assays

IQ® Assays have been run in a variety of plate densities, including 96-, 384-, and 1536-well formats. The instructions for performing an assay include directions for all of these formats to facilitate ease of use. Accurate data have been generated in all formats, and different well densities will produce equivalent results as illustrated in Figures 5A and 5B.

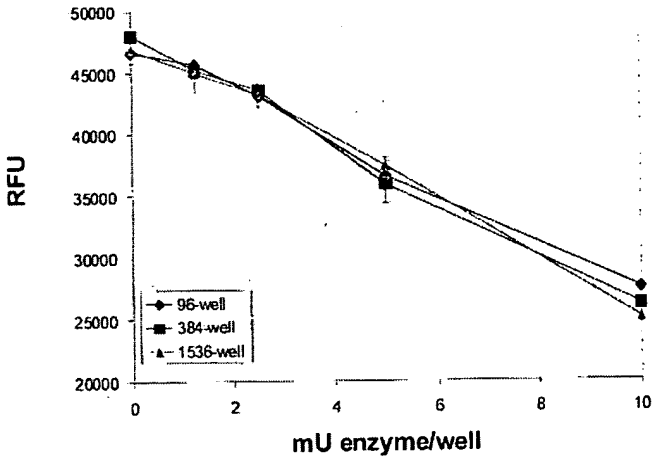

Figure 5A. Src Tyrosine kinase activity in 96-, 384-, and 1536-well formats.

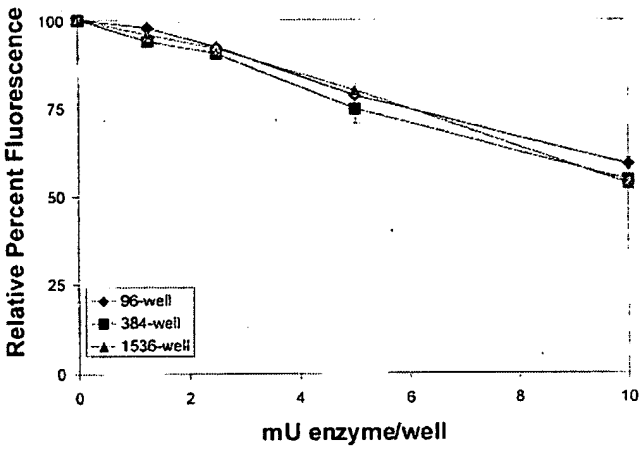

Figure 5B. Conversion of data shown in Figure 5A into relative percent fluorescence using the method described in the Data Management section of this handbook.

Calibrators

The Calibrator Set for IQ® Assays (Product # 62520) consists of a matched pair of dye-labeled peptides that vary only in the amount of phosphorylation. Therefore, each standard contains the same total concentration of the dye-labeled peptides, but the percent phosphorylated to non-phosphorylated peptide differs. The calibrator set may be used for the following applications: 1) as a control for the assay matrix, 2) as a method to test compatibility of specific reaction conditions to use in the IQ® Assay, and 3) as a method to calculate the concentration of a peptide. A typical calibrator curve is illustrated in Figure 6.

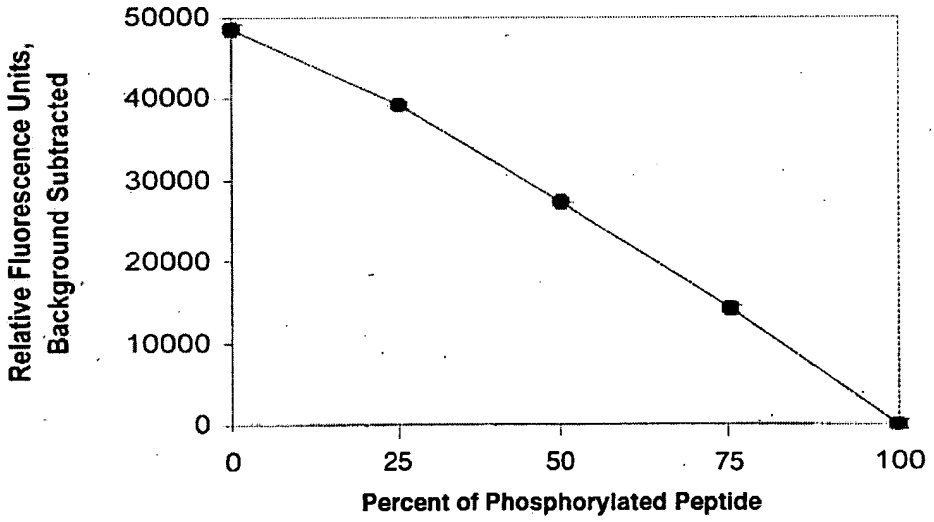

Figure 6. Use of the Calibrator Set for IQ® Assays. The final concentration of peptide used was 60 µM; each point represents the percent phosphopeptide. The background fluorescence at 100% phosphopeptide has been subtracted from the RFU's at each point.

Because a molecule's relative fluorescence changes in the presence of certain organic molecules and detergents, the IQ® Calibrator Set can be used to identify potential differences in RFU caused by any introduced reagents. Converting the data to normalized fluorescence (as described in the Data Management section) can eliminate these differences in RFU. The difference in absolute RFU when the calibrator is diluted with water vs. 5% Triton®-X 100 is indicated in Figure 7A. Detergent increases the relative fluorescence compared to background; however, after converting the data to relative percent fluorescence (Figure 7B) the apparent RFU increase is eliminated.

Normalizing the data cannot compensate for the effects of interfering compounds such as high concentrations of phosphate-containing molecules (Figure 7B). If the data cannot be normalized, the substance is incompatible with this detection method. Please note the IQ® Reagents have been optimized to maintain compatibility with the majority of reaction components that would potentially be used in a kinase or phosphatase assay, including high concentrations of ATP and DMSO.

To test compatibility of a substance, add the specific substance to each of the five different percent phosphorylation concentrations of the IQ® Calibrator Set. Results will indicate whether there is a change in relative fluorescence units that adversely affects the ability to measure differences in percent phosphorylation.

A large number of substances anticipated to be used in a kinase or phosphatase assay have been tested at various concentrations using the IQ® Technology. Refer to the IQ™ Compatible Substances information in this handbook in Tables 2, 3, and 4.

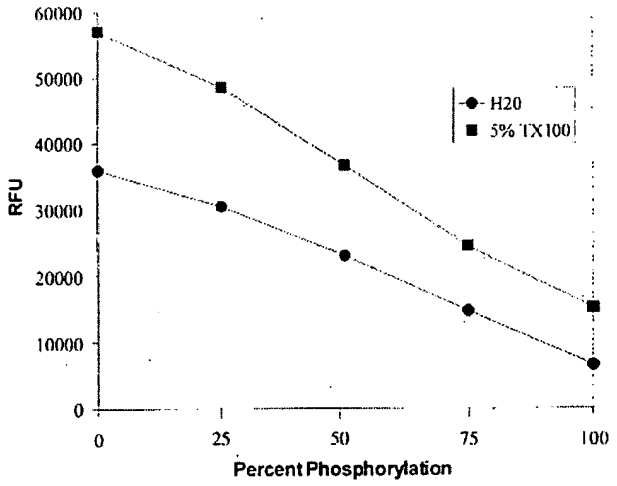

Figure 7A. Comparison of Calibrator Set in water or 5% Triton® X-100.

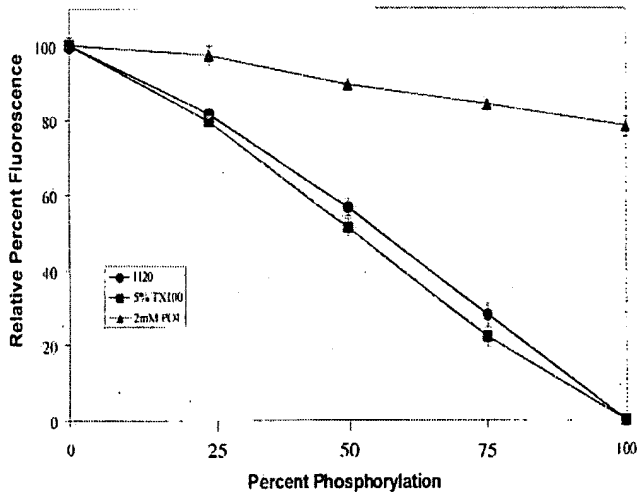

Figure 7B. Normalized data from Figure 7A, with additional data showing the Calibrator Set in the presence of 2 mM phosphate.

IQ® Reagents

IQ® Reagent A and IQ® Reagent B are used to prepare the full-strength (1X) IQ® Working Solution. In an assay, the IQ® Working Solution can be used at 1X strength or it can be further diluted with water to provide 0.5X or 0.25X strength working solutions. The 1X, 0.5X or 0.25X working solutions can be used interchangeably to accurately measure enzyme activity, provided the appropriate volume of IQ® Working Solution to enzyme reaction volume of the chosen IQ® Working Solution (IQ® WS) dilution is utilized (1:1, 2:1, or 4:1; see Table 1). The more concentrated working solutions provide the flexibility to reduce total volume in higher density plate formats. Different concentrations of the IQ® Working Solution were added to the equivalent reaction mixtures, and results are illustrated in Figure 8. Insufficient mixing of the IQ® Reagents will alter the results (Figure 9); however, pipetting the IQ® Working Solution into the reaction mixture will usually provide sufficient mixing of reagents. Deviations from the recommended formulation are not recommended, as it will result in inaccurate activity measurements. The IQ® Working Solution should be transparent; cloudy or precipitated working solutions should be discarded. A 1X IQ® Working Solution can be stored at 4°C for up to 2 months, then diluted to a 0.5X or 0.25X Working Solution prior to use. A 0.5X or 0.25X Working Solution can be stored at 4°C for 2 days.

Table 1. The ratio of IQ® Working Solution to enzyme reaction volume used for optimal results.

	0.25X IQ® WS Volume Ratio	**0.5X IQ® WS** Volume Ratio	**1X IQ® WS** Volume Ratio
IQ® Working Solution	4 parts	2 parts	1 part
Enzyme Reaction	1 part	1 part	1 part

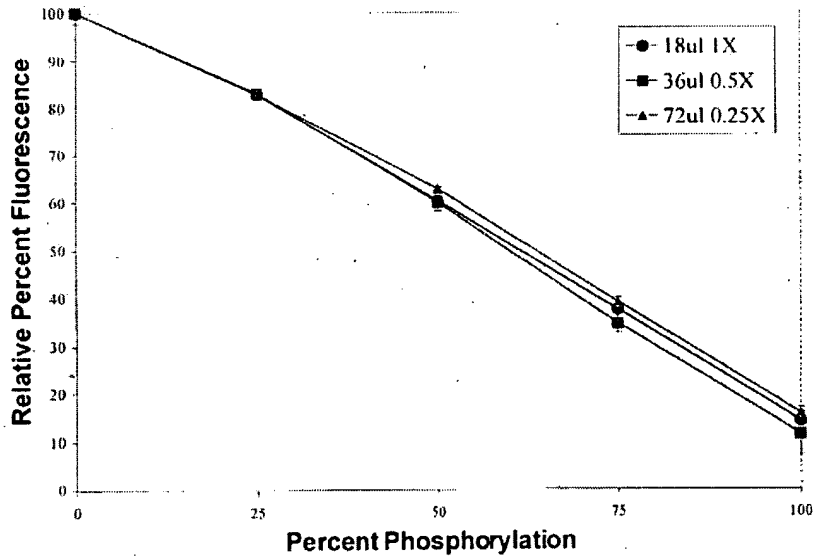

Figure 8. Addition of 72 µl of 0.25X, 36 µl of 0.5X or 18 µl of 1X Working Solution to wells (384-well, white) containing equivalent reaction mixtures. At one gain setting on an instrument, RFU will be different at different volumes, but normalized data results will be equivalent.

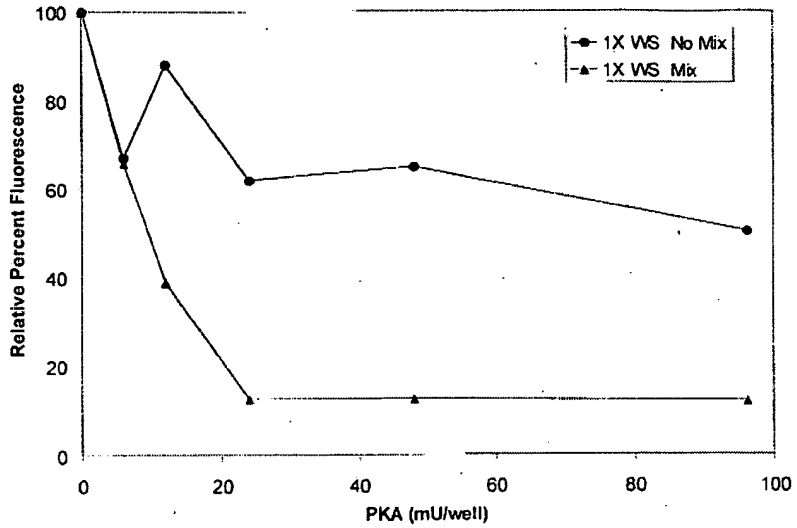

Figure 9. Comparison of mixing versus not mixing the IQ® Working Solution with the assay components after a PKA enzyme reaction was performed in a 384-well plate.

The IQ® Working Solution exhibits a time-dependent color change upon mixing that appears as a darkening of the solution to reddish brown. If the Working Solution is added to a small number of samples and all results determined within a few minutes, then the associated color change will not affect results. However, if the IQ® Working Solution will be used at different times and these samples compared, then the effects of changes in working solution color need to be eliminated. The following methods allow one to use the IQ® Working Solution without effects due to associated color changes: 1) prepare the IQ® Working Solution one day before performing the experiment or 2) warm the IQ® Working Solution for 5 minutes in a 60°C water bath to accelerate completion of the color change process. Multiple experiments can then be performed at different times. The effects of performing the assay at different stages in the color change process are indicated in Figure 10. To determine whether the IQ® Working Solution is functional, a test may be performed using the Calibrator Set.

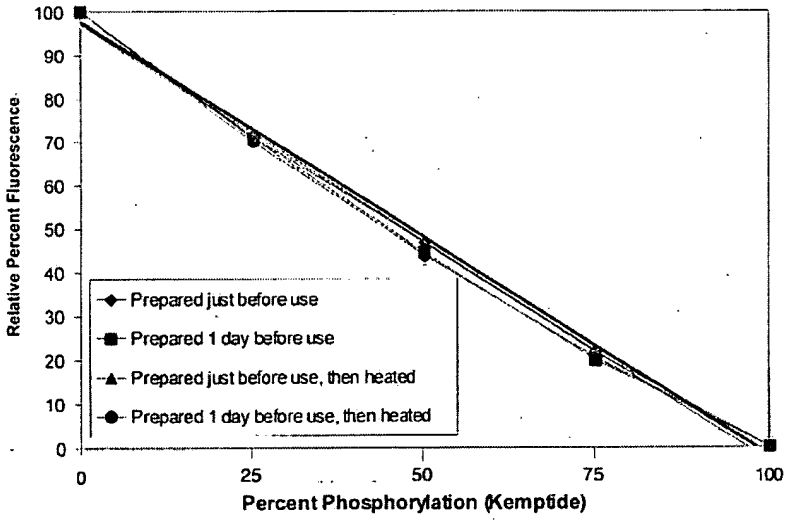

Figure 10. The effects of performing the assay at different stages in the IQ® Working Solution color change process using the Calibrator Set. Data have been normalized by subtracting out the fluorescence at the 100% phosphorylation, then dividing the RFU of each data set by the RFU at 0% phosphorylation.

Relative percent fluorescence remains stable for 48 hours after addition of the IQ® Reagents (Figure 11). Multiple measurements of the same plate may decrease absolute fluorescence intensity caused by photobleaching and evaporation; however, converting the data to relative percent fluorescence will eliminate these differences. The effects of multiple measurements are indicated in Figure 12.

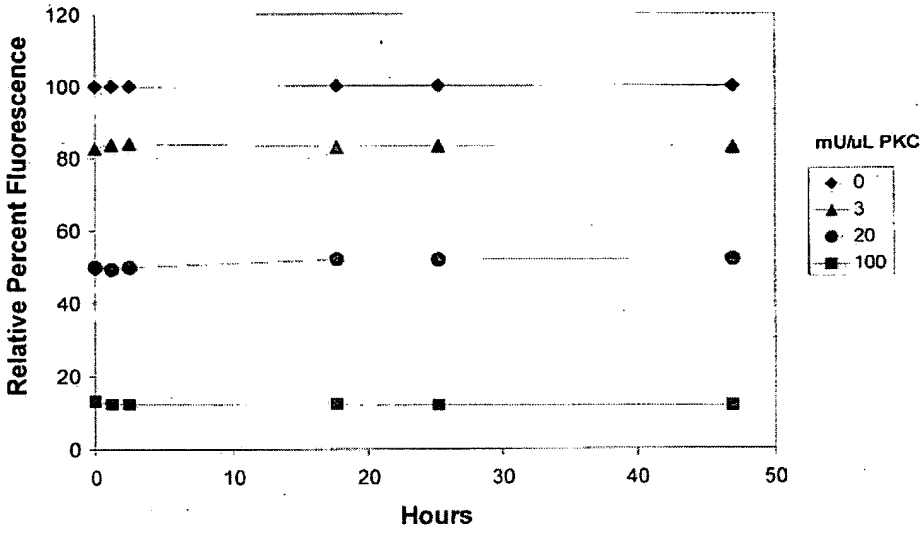

Figure 11. A plate was measured at multiple time points and the data normalized. Relative percent fluorescence did not change during the two days that data were generated. A 0.5X Working Solution was used in this experiment.

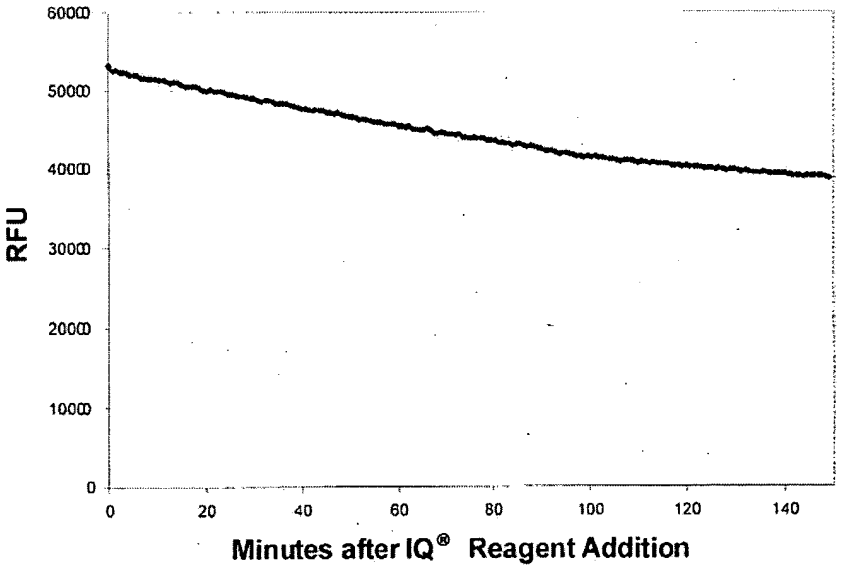

Figure 12. Raw data generated by evaluating one plate at multiple time-points indicates that some photobleaching does occur. However, these differences can be normalized as demonstrated in Figure 11 using the data from Figure 12.

IQ® Assay Compatible Substances Information

Solvents and local environment can affect the emission spectra and quantum yield of fluorophores. To identify which substances affect relative fluorescence intensity using the IQ® Assay Platform, a group of substances was analyzed using multiple dilutions. The substances and specific final concentration in the enzyme reaction are indicated in Tables 2-4. Substances are classified as follows: 1) ***compatible and noninterfering*** refers to substances that do not affect the relative fluorescence units (Table 2); 2) ***compatible*** refers to substances that affect relative fluorescence units, but do not affect the final results when data are normalized (Table 3); 3) ***incompatible*** refers to substances that affect relative fluorescence units differently at different phosphorylation concentrations that cannot be eliminated in the final data (Table 4). However, with proper controls the majority of the effects can be eliminated and will not impact the final results. The IQ® Reagents have been optimized to eliminate incompatibilities of the majority of reagents used in assaying kinases and phosphatases and for storing compound libraries. Please note that each substance was tested individually. The addition of several different substances may have a synergistic effect, so that the interference could occur at a lower concentration than indicated in the chart.

ATP is a vital component of kinase assays and has the potential to interfere with the iron binding because of its phosphate groups. The IQ® Reagents have been optimized to eliminate interferences at up to 10 mM ATP. Note that interferences are additive and 10 mM ATP tests were run with no other potentially interfering substances. Compatibility of the IQ® Technology with high concentrations of ATP is illustrated in Figure 13.

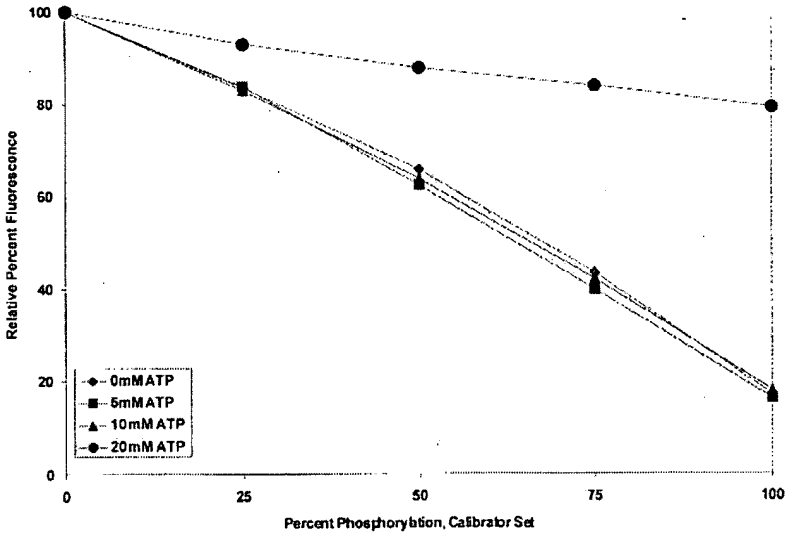

Figure 13. The effects of high concentrations of ATP on detection capacity in the IQ® Assay using the IQ® Calibrator Set.

Compatible Substances Chart

Table 2. Substances that will not change the RFU at the indicated concentrations.

Compound	Compatible Concentration
Acetonitrile	10%
Ascorbic Acid	2 mM
ATP	2 mM
BGG	0.1 mg/ml
Brij®-35	0.10%
Brij®-58	1%
BSA	0.01 mg/ml
$CaCl_2$	10 mM
CHAPS	1%
DMF	5%
DMSO	10%
DTT	2 mM
EDTA	2 mM
EGTA	2 mM
Ethanol	5%
Ethylene glycol	10%
Glycerol	25%
HEPES, pH 7.4	25 mM
Imidazole	2 mM
KCl_2	2 mM
Methanol	5%
$MgCl_2$	20 mM
$MnCl_2$	10 mM
NaCl	100 mM
NaF	1 mM
Na_3VO_4	1 mM
NH_4SO_4	2 mM
Nonidet NP-40	0.10%
Octylglucoside	10%
Octythioglucoside	10%
Peroxide	10%
Sodium Iodate	1 mM
TCEP	2 mM
Thiourea	2 mM
Tris·HCl, pH 7.0	25 mM
Tris·HCl, pH 8.0	25 mM
Triton® X-100	0.10%
Triton® X-114	0.10%
Tween®-20	1%
Tween®-80	1%
Urea	30%
$ZnCl_2$	2 mM

Table 3. Substances that will change the RFU at the indicated concentrations but will not affect the final results when data are normalized to percent fluorescence.

Compound	Compatible Concentration
ATP	10 mM
Brij®-35	10%
Brij®-58	10%
BSA	0.1 mg/ml
CHAPS	10%
DMF	10%
DMSO	15%
DTT	10 mM
Ethanol	10%
Ethylene glycol	15%
Methanol	10%
Nonidet NP-40	10%
Triton® X-100	10%
Triton® X-114	10%
Tween®-20	10%
Tween®-80	10%

Table 4. Substances that will interfere with IQ® Technology at the indicated concentrations.

Compound	Interfering Concentration
ATP	20 mM
β-glycerolphosphate	2 mM
BGG	1 mg/ml
BSA	1 mg/ml
Hemoglobin	1 mg/ml
Phosphate	2 mM
Sodium citrate, pH 5.5	25 mM
TFA	0.10%

General Determination of Enzyme Assays using IQ® Technology

Performing an enzyme assay using the IQ® Technology as a detection method is essentially the same as using other detection methods. Initially the researcher needs to determine both the enzyme concentration to use in the assay and the time length to perform the reaction. The ideal reaction conditions are when the substrate to enzyme mole ratio is approximately 5:1 or greater and the initial slope rate of product vs. time or substrate vs. time is linear (generally less than 20% substrate depletion). To determine the enzyme concentration to use, the linear reaction time and minimum enzyme needed to obtain measurable generation of product, perform the following experiment: 1) Start with the recommended concentration of substrate and calculate an amount of enzyme approximately two-fold less in concentration than the substrate. 2) Prepare a reaction in which the enzyme is diluted out over several concentrations. 3) For each concentration of enzyme, remove an aliquot at multiple time points, such as at 15, 30, and 45 minutes, and stop the reaction by adding the IQ® Working Solution (see Figure 14). The IQ® Technology cannot be used for real time kinetic studies, because the addition of the IQ® Working Solution changes the pH of the solution to 5.5.

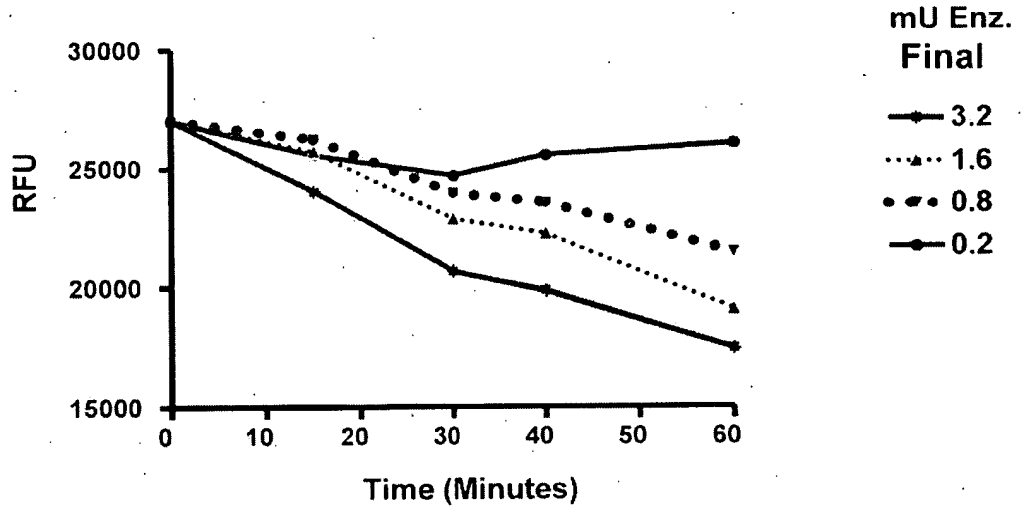

Figure 14. Time-course determination for PKC reaction. Different concentrations of PKC were diluted into a reaction mixture. At set time points an aliquot was removed, placed in a well and IQ® Reagents were added. The plate was then read and RFU graphed versus time.

Another useful parameter that can be generated using the IQ® Assay is determination of the K_m. The K_m is the substrate concentration at which the rate is half-maximal (when v=1/2 Vmax). The K_m can be determined by using a large range of diluted substrate and performing the reaction at a constant enzyme concentration for a set time course (already determined to be linear in rate at the lowest substrate concentration used). Because the peptides used in the IQ® Assay have a dye attached, the K_m is generally higher than the free peptide. The K_m for dye-labeled pseudosubstrate was generated vs. PKC and is indicated in Figure 15.

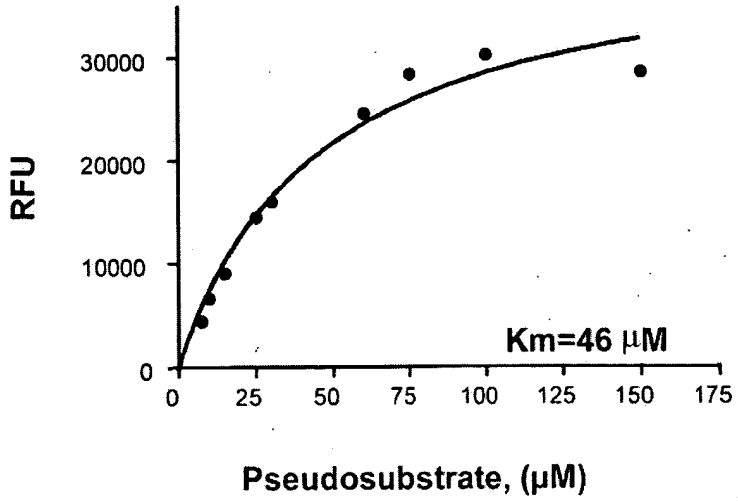

Figure 15. Pseudosubstrate was titrated at multiple concentrations and reacted with PKC for 30 minutes. The K_m was generated using nonlinear regression analysis on Graphpad Prizm.

Kinases recognize their substrates based on the sequences surrounding the serine, threonine or tyrosine, which they phosphorylate. The sequence requirements, or motif recognition sites, are often recognized by multiple kinases. The peptide called Pseudosubstrate, based on the pseudosubstrate site of PKC, contains specific amino acids in a motif that is recognized by many kinases. Therefore, it may be useful to measure activity of kinases other than just the PKCs. For example, PKB and PKG both recognize this motif and have both shown activity with Pseudosubstrate and Kemptide substrates, which contain a common motif. The activity of these enzymes in the presence of pseudosubstrate is indicated in Figure 16. Another peptide that is generally used to measure PKB activity is the Crosstide peptide, and this peptide has been used with a dye-label in the IQ® Kinase Assay with excellent results.

The concentration of peptide needed for a kinase assay will not affect the detection component of this assay, because the IQ® Reagents are not affected by total peptide concentration. High micromolar to low nanomolar concentrations of peptide will be measurable in this assay from the perspective of fluorescence intensity measurement. The primary limitation will be the sensitivity of the instrument. The kinase requirements will need to be determined empirically. Three different peptide concentrations in which the normalized data are equivalent is illustrated in Figure 17.

The distance between the fluorescent dye and the phosphate group on the peptide may be as long as 20 amino acids. Longer distances may work but have not been tested. The data are illustrated in Figure 18.

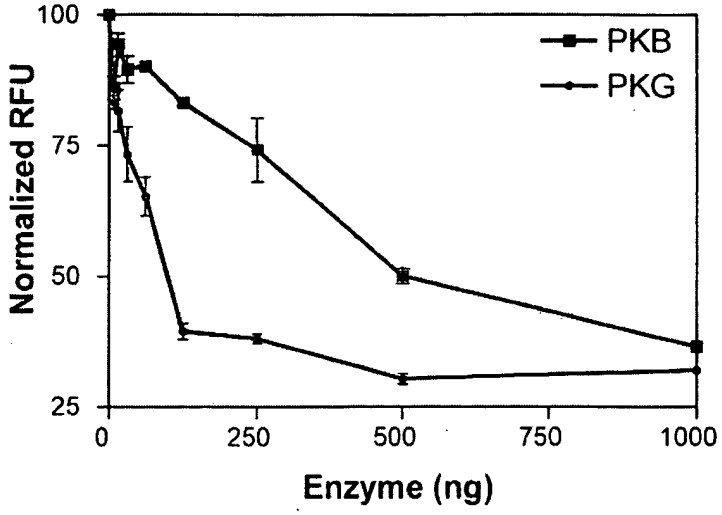

Figure 16. PKG or PKB was serially diluted into a reaction mixture containing the peptide pseudosubstrate. The differential phosphorylation of the peptide at each concentration of enzyme was measured.

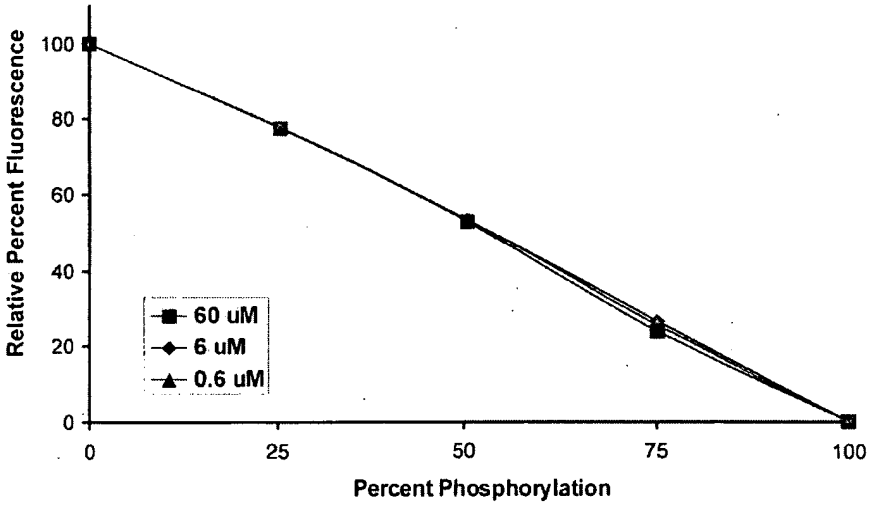

Figure 17. The IQ® Calibrator Set was analyzed at three different peptide concentrations as indicated. Lower peptide concentrations can also be utilized. Relative percent fluorescence does not change.

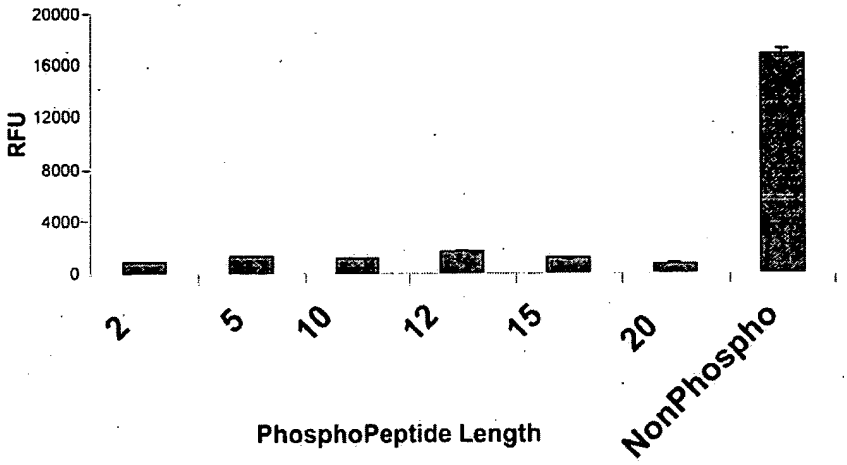

Figure 18. Analysis of distance-dependence between the phosphoryl group and the dye-label. Various length peptides based on the kemptide sequence were synthesized in which the distance from the phosphoryl group to the dye-label was increased to determine the maximum distance in which quenching would occur. The X-axis represents the distance between the dye and the phosphate group on the peptide. The Nonphospho-peptide does not have a phosphoryl group; therefore, the IQ® Reagents do not bind to it. Peptides may be longer than those indicated, as long as the dye to phosphoryl group distance is maintained.

Generation of inhibition curves is important when trying to identify molecules that change an enzyme's activity. In brief, when trying to identify a potential inhibitor (or activator) of a kinase, prepare multiple dilutions of the compound using a large range of concentrations. Perform the reaction using conditions that are known to be within steady state kinetic parameters. The resulting data may be converted to percent relative fluorescence and an IC_{50} curve generated as indicated in Figures 19 and 20. Comparison of these data to previously reported data is also shown in Table 5 to indicate the viability of the IQ® Technology.

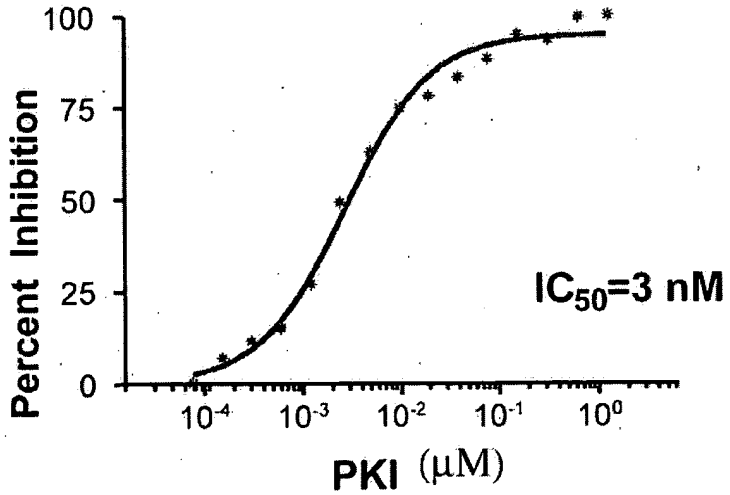

Figure 19. Inhibition of PKA by PKI, a peptide inhibitor of the substrate binding site. PKA was incubated with the indicated concentration of PKI, plus 60 uM dye-labeled kemptide, 100 uM ATP, 20 mM Hepes, 5 mM $MgCl_2$, pH 7.4, for one hour prior to the addition of IQ® WS. Data were analyzed using nonlinear regression analysis, and the results were normalized and converted to percent inhibition.

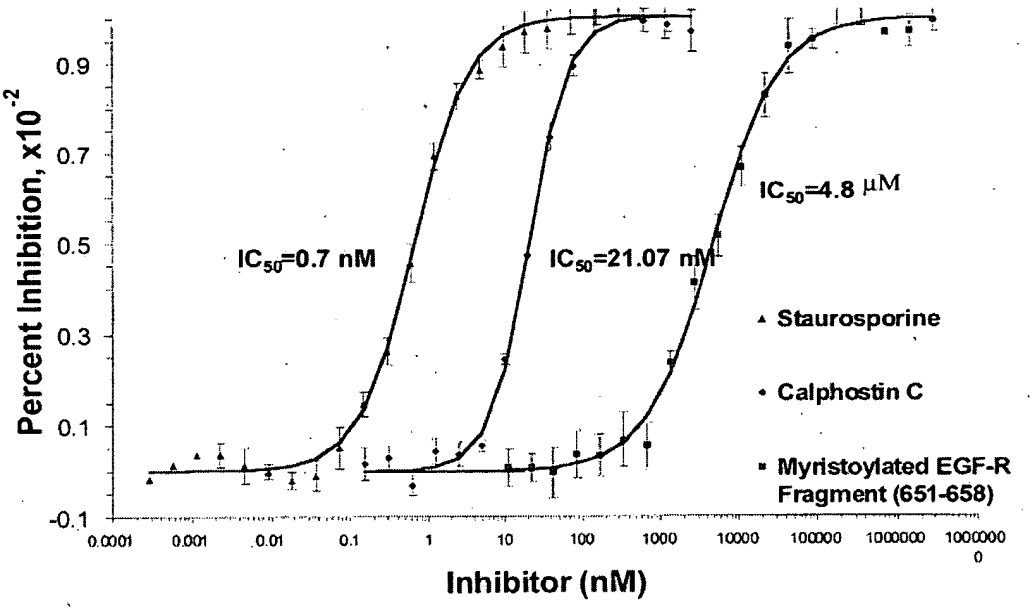

Figure 20. Inhibition curves were generated using the IQ® Reagents using 500 µU PKC per ml of final enzyme reaction mixture pre-incubated with the listed inhibitor, then incubated with 60 µM p seudosubstrate for 1 hour. Fluorescence was measured and data were analyzed using nonlinear regression analysis.

Table 5. IC_{50} values experimentally found with IQ® Assay compared to previously reported values.

PKC Inhibitors	Best Fit	95% confidence interval	Previously Reported*
Staurosporine	0.6683 nM	0.6090 to 0.7334 nM	0.7 nM
Chelerythrine Chloride	524.1 nM	459.8 to 597.4 nM	700 nM
Myristoylated Protein Kinase C Inhibitor 20-28, Cell-Permeable	8.367 uM	7.230 to 9.682 uM	8.0 uM
Myristoylated EGF-R Fragment (651-658)	4.837 uM	4.199 to 5.571 uM	5.0 uM
Bisindolylmalemide I	20.21 nM	17.56 to 23.26 nM	10 nM
Calphostin C	20.95 nM	19.45 to 22.56 nM	50 nM

* Kuo, J.F. (1994) Protein Kinase C Oxford University Press: New York, NY.

Statistical Parameters for IQ® Assays

The Z' is a statistical parameter, which was referenced in the Journal of Biomolecular Screening (1999), is used by the drug screening community to evaluate and validate high-throughput screening assays. The Z' for the IQ® Kinase Assay was generated using the precision of the 0% phosphorylation signal and 10%, 15% or 20% phosphorylation signal, and the magnitude of the displaced signal according to the following equation:

$$Z' = 1 - \frac{(3 \times \sigma 0\%) + (3 \times \sigma 10, 15, \text{or } 20\%)}{|\text{mean RFU } 0\% - \text{mean RFU } 10, 15, \text{or } 20\%|}$$

In this equation, σ = standard deviation. The denominator of the fraction represents the displaced signal. Values of $Z' > 0.5$ indicate excellent assays. An example Z' for the IQ® PKA Assay at 10%, 15% and 20% is represented in Figure 21. Experimental Z' values were 0.6, 0.7 and 0.8, respectively, for 10%, 15% and 20% product formation. As may be evident from the graph, one can improve the Z' merely by choosing a different percent product formation.

Signal- to-noise and signal-to-background values for the IQ® Assay were generated using the following equations:

$$S/N = \frac{\left(\overline{RFU}_0 - \overline{RFU}_{sample}\right)}{\sqrt{(\sigma_0)^2 + (\sigma_{sample})^2}} \qquad S/B = \frac{\overline{RFU}_0 - \overline{RFU}_{sample}}{\overline{RFU}_0 - \overline{RFU}_{100\%}}$$

Table 6. These are representative signal-to-noise and signal-to-background values generated from IQ® Kinase Assays.

S/N - Average	S/B - Average
10% conversion: 10	10% conversion: 5
15% conversion: 16	15% conversion: 8
20% conversion: 17	20% conversion: 10

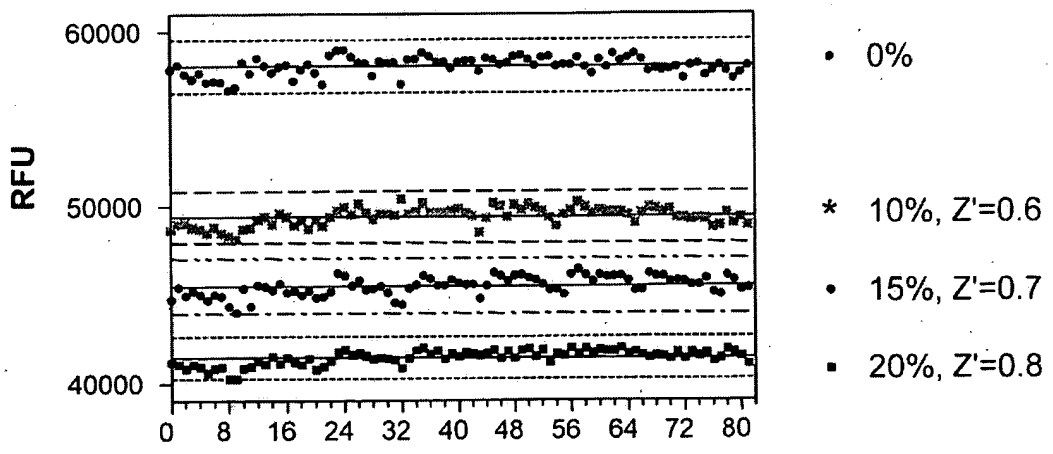

Figure 21. IQ® Kinase Assay variability. A PKA kinase assay reaction was stopped at 10%, 15%, and 20% turnover using 90 replicates for each. Peptide concentration was 30 uM final. The Z' was determined using the equation shown (Zhang, *et al., Journal of Biomolecular Screening*, 1999). Z' values generated were 0.6, 0.7, and 0.8 at 10%, 15%, and 20% substrate turnover. For phosphatase assays, Z' values for 5%, 10%, and 15% dephosphorylation were 0.7, 0.7, and 0.8 (data not shown).

Data Management

Most fluorometers are driven by software that generates raw data as relative fluorescence units (RFU). Statistical analysis and graphical representation of these data are accomplished by exporting the raw data file to a standard spreadsheet application. Data, generated from an IQ® Assay of either kinase or phosphatase activity, can be graphed as RFU vs. enzyme, agonist or antagonist concentration. When assessing variable dye-labeled peptide concentrations or measuring multiple plates with variable instrument gain settings, it is recommended that the raw data to be normalized to 100-0% activity for kinase assays and 0-100% activity for phosphatase assays.

Data for the IQ® Kinase Assay is first corrected for the background RFUs of the 100% enzyme activity well. The 100% enzyme activity well is the data from the 100% phosphorylated peptide in the Calibrator Set. The RFU of the 100% enzyme activity well is subtracted from the RFU of each test well including the no enzyme control. Each corrected RFU is divided by the corrected RFU of the no enzyme control and multiplied by 100 for a percentage. The no enzyme control is now the 100% RFU and the 100% enzyme activity is the 0% RFU. Alternatively, data may be normalized so that all experimental wells are related to the percentage of RFU (such as 100-0%) relative to a negative control (no enzyme or no inhibitor control). This does not take into account the background fluorescence of the 100% phosphorylated (theoretical 100% quenched) well. However, in most instances the data normalized by this method will not vary significantly from data normalized using the method that also incorporates the background RFU subtraction.

Data for the IQ® Phosphatase Assay is normalized in a similar manner as kinase assay data, but relative to the opposite enzymatic activity being assayed. The data is first corrected for the background RFUs of the no enzyme control well. In the phosphatase assay 100% enzyme activity is the non-phosphorylated peptide from the Calibrator Set. The RFU of the no enzyme control well is subtracted from the RFU of each test well including the 100% enzyme activity well. Each corrected RFU is divided by the corrected RFU of the 100% enzyme activity well and multiplied by 100 for a percentage. The no enzyme control is now the 0% RFU and the 100% enzyme activity is the 100% RFU.

A normalized data set as illustrated in Figure 22A and 22B is a valuable tool for comparing the results of independent experiments in the IQ® Assay format.

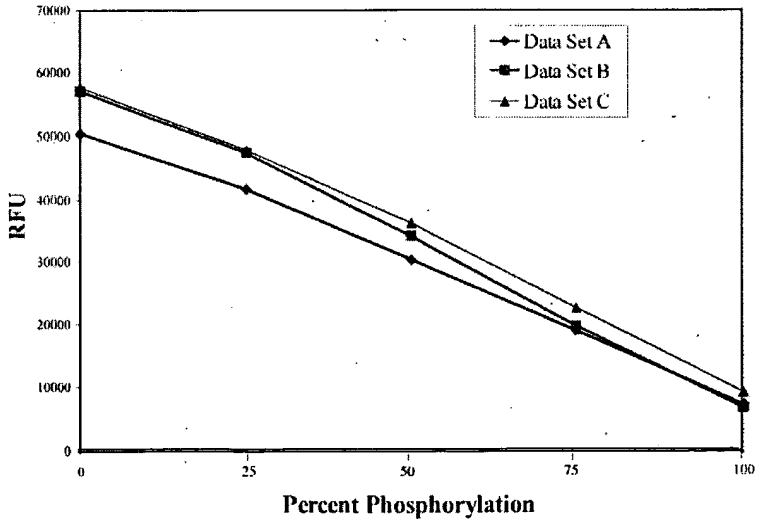

Figure 22A. Three sets of data were generated using the Calibrator Set and the RFU were graphed.

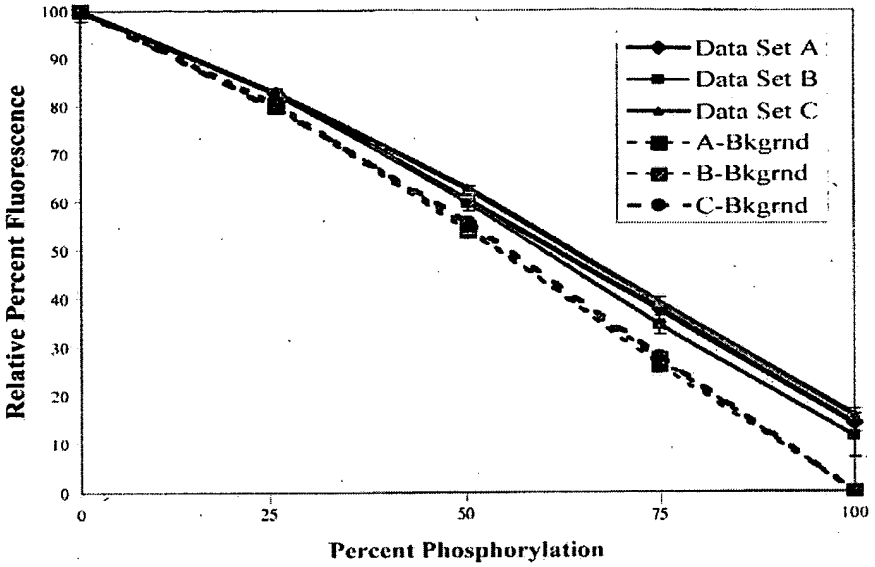

Figure 22B. RFU from Figure 22A were converted to relative percent fluorescence without background fluorescence at the 100% phosphorylation data point subtracted (solid lines) or with background fluorescence at the 100% phosphorylation data point subtracted (dashed lines).

IQ® Technology for Phosphatase Screening

The data presented in this handbook have been generated primarily using kinase reactions. The IQ® Technology can also be used to detect activity of phosphatases, based on its specificity for phosphate groups. The following data (Figures 23A, 23B, 23C and 23D) show both serine/threonine phosphatase and tyrosine phosphatase data using their respective substrates.

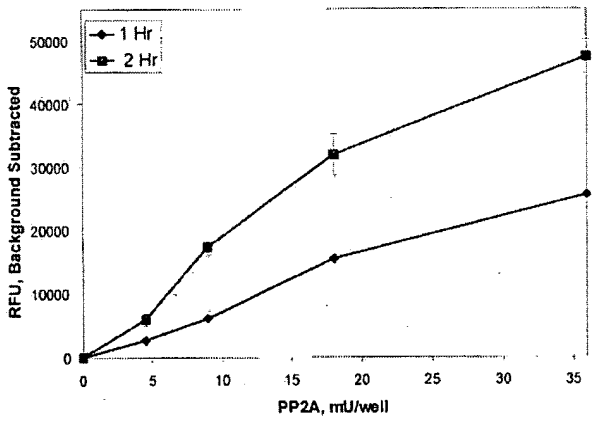

Figure 23A. PP2A Phosphatase activity was measured in the presence of the peptide substrate, LRRApSLG. Several concentrations of enzyme and two timepoints were analyzed.

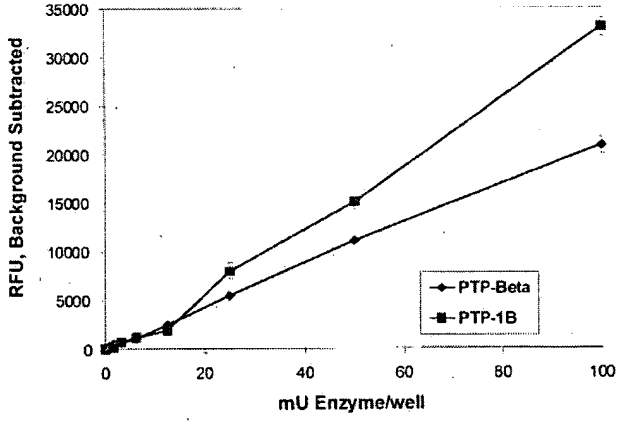

Figure 23B. PTPBeta or PTP1B Phosphatase activity was measured in the presence of the peptide substrate, KVEKIGEGTpYGVVYK. Several concentrations of enzyme were analyzed.

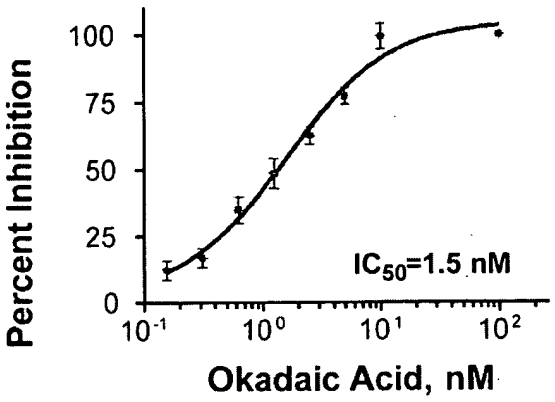

Figure 23C. PP2A Phosphatase activity was measured in the presence of the phosphopeptide substrate, LRRApSLG versus increasing concentrations of the inhibitor Okadaic Acid.

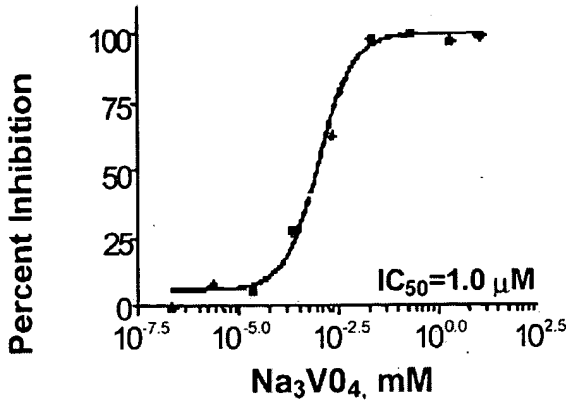

Figure 23D. PTP1B Phosphatase activity was measured in the presence of the phosphopeptide substrate, KVEKIGEGTpYGVVYK.

Troubleshooting

Problem	Cause	Solution
No enzyme-dependent fluorescent quenching observed	Insufficient kinase activity	Increase time of incubation and/or amount of kinase **Note:** If the kinase storage buffer contained EDTA and/or EGTA, confirm that the selected dilutions reduced the amount of chelators to less than 0.1 mM
	Phosphatase contamination	Add sodium vanadate to the reaction buffer
Experimental samples result in higher RFU than a standard curve using purified enzyme	High concentrations of detergents can increase the apparent quantum yield of the fluorophore	Include detergent in the enzyme standard curve at the same concentration as the test samples to avoid matrix effects
No fluorescence observed	Improper excitation/emission filters and/or gain setting	Check instrument setup parameters
High coefficients of variation	Pipetting variation, temperature variation, variable evaporation, edge effects	Consider experimental design to minimize variability
Partial sample evaporation	Lack of humidity control, uncovered microplate during enzyme reaction	Partial sample evaporation is tolerated by the IQ® Assays; ensure that evaporated portion is re-dissolved after IQ® Working Solution addition by mixing
High and constant RFU consistent with photomultiplier tube saturation	Inappropriate excitation/emission filters	Refer to transmission profiles of the instrument filters for minimal cross-talk of the excitation light or limit emission of the fluorophore by using off-peak illumination or narrower band pass filters
Insufficient delta RFU	Enzyme concentration is too low	Use more enzyme
	Incubation time was too short	Increase incubation time
	Inner filter effects associated with high peptide concentrations	Use off-peak illumination or decrease peptide concentration

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